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(57) Abstract

Animal cells which are totipotent for nuclear transfer (TNT cells) can be isolated from the embryonic disc of the blastodermic vesicle of an ungulate, or the equivalent tissue of an embryo at an equivalent stage in non-ungulate species. TNT cells can be cultured *in vitro*, which allows cloning as desired and genetic manipulation, for example to introduce a transgene. Their nuclei can be transferred to suitable recipient cells and embryos reconstituted. In this way, animals of high genetic merit may be cloned and transgenic animals may be generated by mass transformation techniques across a broader range of species than is accessible with embryonic stem cell technology and without reliance on pronuclear microinjection.

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TOTIPOTENT CELLS FOR NUCLEAR TRANSFER

This invention relates to the generation of animals, including but not being limited to transgenic animals, and to cells useful in their generation.

The cloning and propagation of cells capable of developing into healthy animals are objectives which have been sought for some time by animal breeders and by producers of transgenic animals. Animal breeders dealing with non-transgenic animals have long sought a means of cloning animals of high genetic merit. The nature of such merit will of course depend on the objectives of the breeder, but it is clear that the dairy industry, to take one example, would benefit from the ability to limit births of calves to those of a single sex.

Gene transfer (transgenesis) has been widely used in the mouse to address questions of gene function, and more sparingly in domestic species, in attempts to alter characters with high economic value. Currently, whole animal transgenesis in species other than mouse can only be achieved by pronuclear injection or, less commonly, by viral transfection. A method from transgenic cultured cells in the mouse is also available, known as the embryonic stem cell (ES cell) system. This system depends upon the isolation in culture of a specific embryonic lineage which may be modified *in vitro* while retaining its unique ability to participate in development following transplantation as an intact cell, to early embryos. Proven ES cells are not available in species other than mouse and ES cell nuclei do not support mouse development when transferred to enucleated zygotes or oocytes using present procedures.

Although a number of abstracts over the years have reported th establishment of lines of pluripotent embryonic cells derived from morula or blastocyst stage embryos of ungulates, two of the most recent being Stice *et al.*, *Theriogenology* **41** 301 (1994) and Strelchenko & Stice, *Theriogenology* **41** 304 (1994), there is as yet no evidence that permanent ES-like cell lines in ungulates can give rise to healthy animals.

Pronuclear microinjection is currently the only practicable procedure for gene transfer in ungulates, particularly farm animals. The efficiency of transgenesis in these species suffers due to the granular nature of the cytoplasm which renders it difficult to visualise pronuclei and because of the smaller number of eggs which may be obtained per animal. Additional constraints are the huge cost of animals and the need, in cattle, to transfer single embryos to each recipient female in order to avoid freemartins.

In contrast to gene transfer, which is much easier in mice than in farm animals, nuclear transfer (NT) in farm animals has been relatively more successful than in the mouse. Calves and lambs have been generated using nuclei from cells of the blastocyst inner cell mass in both cattle (Keefer *et al.*, *Biol. Reprod.* **50** 935-939 (1994) and Sims & First, *Proc. Nat'l. Acad. Sci. USA* **90** 6143-6147 (1994)) and sheep (Smith & Wilmut, *Biol. Reprod.* **40** 1027-1035 (1989)). Similar experiments in mice are controversial, but have probably not yielded pregnancies.

Embryonic stem cells (ES cells) are tissue culture cells isolated from the inner cell mass of the mouse blastocyst which retain in culture their ability to participate in

normal development. This capacity is dramatically demonstrated when ES cells are returned to the early embryonic environment, wherein they participate in normal development, giving rise to chimeric animals whose tissues are a mosaic of host embryo and ES cell genotypes. Where ES cells contribute to primordial germ cells, then genetic manipulations to ES cells *in vitro* can be passed on to transgenic animals. There has been much interest in the isolation of ES cells from farm animals due, largely, to the expectation that ES nuclei might be competent for nuclear transfer, offering a more efficient route to transgenesis. There is, however, no evidence that competence for NT is a property of ES cells.

ES cells are defined by their ability to make germline chimeras. In the development of transgenic farm animals, because of the opportunities for pre-transfer screening and gene targeting and to avoid an extra chimeric generation, there is more interest in achieving transgenesis through nuclear transfer from cultured cells. The present invention, therefore, arises from the idea that an efficient gene transfer system through NT from cultured cells may be achieved without the necessity of isolating ungulate ES lines.

Sims & First (*Proc. Nat'l. Acad. Sci. USA* **90** 6143-6147 (1994)) describe the production of calves by transfer of nuclei from cultured inner cell mass (ICM) of bovine blastocysts. However, based on the data given in this paper, there is no evidence of the establishment of a true cell line from the ICM cells. For example, the authors state:

> ICM cells from day 9 and 10 bovine blastocysts multiplied in culture when culture in CR1aa

> plus SIT and 5% FCS with some lines reaching 2,000 cells after 2 weeks of culture. [page 6144, right hand column, lines 40-43]

2,000 is a remarkably low number of cells for anything that could reasonably be called a cell line. A single confluent 25 cm flask of ES cells, for example, would generally contain 10^6 to 10^7 cells, and a line would grow to 10^9 to 10^{10} cells after five or six passages.

In fact, it is clear that Sims & First do not use conventional passaging techniques: instead of attaching cells to a tissue culture substrate and detaching and replating the cells when they reach confluence, as is traditional, the authors maintained the ICM cells in suspension culture for up to two months. If no more than 2,000 cells resulted from this process, little cell division can have been taking place.

It would be desirable to establish a genuine cell line of cells which are totipotent for nuclear transfer. The present invention achieves that goal and is based on the discovery that cells derived from the embryonic disc of blastodermic vesicles can be used to establish such lines.

According to a first aspect of the present invention there is provided an animal cell line derived from an embryonic disc of an ungulate blastodermic vesicle, or the equivalent tissue of an embryo at an equivalent stage in non-ungulate species, cells of which cell line are totipotent for nuclear transfer.

Such cells, designated in this specification as TNT cells, can be isolated, individually or collectively, and themselves form part of the invention, according to a

second aspect of which there are provided isolated animal cells which are derived from an embryonic disc of an ungulate blastodermic vesicle, or the equivalent tissue of an embryo at an equivalent stage in non-ungulate species, and which are totipotent for nuclear transfer.

Stages of non-ungulate embryonic development which are equivalent to the blastodermic vesicle in ungulates are those at or immediately after the determination of the three germ layers at gastrulation; examples include the early egg cylinder stage in rodents and the early gastrula in avian species. Tissues from these stages which are equivalent to the ungulate embryonic disc are those which exclude extra-embryonic lineages; examples include embryonic ectoderm plus visceral endoderm in rodents and embryonic disc in avian species. Where, in the relevant species, embryonic ectoderm can be dissected free of endoderm, embryonic ectoderm alone is the preferred tissue.

Totipotentiality is the capacity of a cell to differentiate into all cell types; it is a property of both nucleus and cytoplasm. In this specification, a cell is said to be "totipotent for nuclear transfer" or "TNT" if, following nuclear transfer from that cell to an oocyte, a healthy animal develops to term. Nuclear transfer may be achieved by fusion of the TNT cell with an oocyte, zygote or early (for example, two cell) blastomere. In the case of the present invention, totipotentiality is a property of the TNT nucleus and the recipient cell cytoplasm. It is to be emphasised, though, that no special properties of the recipient cell are required: any normal (generally enucleated) oocyte, zygote or early blastomere will suffice. Enucleation may

be achieved physically, by actual removal of the nucleus, pro-nuclei or metaphase plate (depending on the recipient cell), or functionally, such as by the application of ultraviolet radiation or another enucleating influence.

Cell lines of the invention can be passaged by conventional means and can be kept in permanent culture. By "permanent" culture is meant culture in which significant reproduction of the cells take place and which can be propagated by passaging; the culture is not necessarily kept indefinitely, but can certainly survive for more than ten passages, by which time it would be regarded as permanently established by those skilled in the tissue culture methodology. At that stage, approximately 10^9 to 10^{10} or more cells may be present in the culture.

In principle, the invention is applicable to all animals, including birds such as domestic fowls. In practice, however, it will be to (non-human) mammals, particularly placental mammals, that the greatest commercially useful applicability is presently envisaged. TNT cells and cell lines derived from small mammals such as rabbits and rodents, especially mice and rats, may be useful in some applications, but it is with ungulates, particularly economically important ungulates such as cattle, sheep, goats, water buffalo, camels and pigs that the invention is likely to be most useful, both as a means for cloning animals and as a means for generating transgenic animals.

Isolation of TNT cells from embryos arising from selected matings among elite stock could be used to clone animals of one or more desired genetic characteristics or high genetic merit generally. This would include the

capability of limiting births to a single sex, which as previously mentioned is of particular importance in the dairy industry.

For the generation of transgenic animals, TNT cells can be genetically manipulated. Then, by a process of nuclear transfer, which in itself is known (see, for example, Campbell *et al.*, *Biol. Reprod.* **50** 1385-1393 (1994)), transgenic animals may be produced from the genetically altered, cultured cells. The overall procedure is expected to have several advantages -- particularly in the generation of transgenic farm animals -- over conventional procedures for generating transgenics, namely (1) that fewer recipient animals will be required, (2) that multiple syngeneic founders may be generated using clonal TNT cells, and (3) that the system will permit subtle genetic alteration by gene targeting.

It should be noted that the term "transgenic", in relation to animals, should not be taken to be limited to referring to animals containing in their germ line one or more genes from another species, although many transgenic animals will contain such a gene or genes. Rather, the term refers more broadly to any animal whose germ line has been the subject of technical intervention by recombinant DNA technology. So, for example, an animal in whose germ line an endogenous gene has been deleted, duplicated, activated or modified is a transgenic animal for the purposes of this invention as much as an animal to whose germline an exogenous DNA sequence has been added.

According to a third aspect of the invention, there is provided a process for the preparation of an animal, the

process comprising reconstituting an animal embryo by nuclear transfer from a TNT cell as described above, allowing the embryo to develop to term and optionally breeding from the animal so formed.

In embodiments of this aspect of the invention in which the animal is transgenic, TNT cells may be genetically modified prior to nuclear transfer. Although micro-injection, analogous to injection into the male or female pronucleus of a zygote, may be used as a method of genetic modification, the invention is not limited to that methodology: mass transformation or transfection techniques can also be used.

By way of illustration, the following scheme sets out a typical process by which transgenic animals may be prepared. The process can be regarded as involving five steps:

(1) isolation of TNT cells;
(2) transgenesis, for example by transfection with suitable constructs, with or without selectable markers;
(2a) optionally screen and select for stable integrants - skip for microinjection;
(3) embryo reconstitution by nuclear transfer;
(4) culture, *in vivo* or *in vitro*, to blastocyst;
(4a) optionally screen and select for stable integrants - omit if done at 2a;
(5) transfer to final recipient.

Isolation of TNT cells

TNT cells can be isolated from explants of the embryonic disc of animals. More particularly, TNT cells can be

isolated from explants of the embryonic discs of arly blastodermic vesicles, for xample at days 9 and 10 in sheep, and at equivalent stages in other animals. For cattle and pigs, the equivalent stage would be days 11 and 12 of the blastodermic vesicle, and in rodents the equivalent would be days 5 and 6 of the egg cylinder.

The most successful procedure for the isolation of TNT cells, which itself forms a fourth aspect of the invention, comprises explanting at least part of the embryonic disc of an animal embryo at the blastodermic vesicle stage in the case of an ungulate, or the equivalent tissue of an embryo at an equivalent stage in non-ungulate species, allowing the development of an ES-like colony of undifferentiated cells until they have acquired an enlarged, more epithelial phenotype (which will usually be by about passage 2) and entering a cell or cells from such a colony into reproductive culture until required.

In practice, substantially the whole of the embryonic disc, essentially free of trophectoderm, will usually be taken, for example by microdissection or immunosurgery, from the embryo. Explantation will be onto a suitable tissue culture substrate or medium, such as a primary mouse fibroblast feeder layer (or other inactivated feeder) layer in ES medium, or with conditioned or supplemented medium. Approximately 10% of explants have in practice been found to give rise to the ES-like colonies of undifferentiated cells, which by passage 2 have acquire the enlarged, more epithelial phenotype referred to above. Such a colony, or at least one or more of the cells from it, may be entered into permanent culture, as shown for example in Example 1 below. TNT

cells from the sheep at passage 3 support development of lambs to term following nuclear transfer to enucleated oocytes (see Example 2 below).

The TNT strategy differs from the ES strategy in that cell lines are isolated from later stage blastodermic vesicles (days 9 and 10 in sheep) and are not required to generate chimeras. TNT cells do not display the classic ES phenotype of small, rounded, undifferentiated cells and are more epithelial in character, growing as a flat monolayer.

The TNT strategy of the present invention also differs from that of Sims & First (*Proc. Nat'l. Acad. Sci. USA* 90 6143-6147 (1994)) in that they isolated cells at an earlier stage of development from the inner cell mass of the blastocyst, rather than the embryonic disc of the blastodermic vesicle, and did not appear to have established true cell lines.

Transfection and nuclear transfer

It is possible to introduce transgene constructs, which may be selectable, by a variety of methods. This is an advantage over the technique of Sims & First: the absence of true cell lines in their paper indicates that their technique will probably be restricted to microinjection; this does not represent much of an improvement over existing pronuclear microinjection techniques. Since the present invention enables the establishment of a line of rapidly dividing cells in culture, mass transformation or transfection techniques can be used, including electroporation, viral-mediated transfection and lipofection. Further, since so many cells are present, the present invention enables

investigators to capitalise on events which happen at low frequency: an exampl of a low frequency event is homologous recombination, which can be harnessed for gene targeting.

If a selectable genetic construct is used, the transfection or transformation step can be followed by culture in selective medium. The invention is not limited to the use of any particular technique for the introduction of a transgene: the foregoing examples are given merely by way of illustration. Using microinjection, it should be possible to introduce transgenes at passage 2 or 3 without selection. Injected DNA integrates stably at a frequency of about 20% in cultured cells (Lovell-Badge, "Introduction of DNA into embryonic stem cells" In: Teratocarcinomas and Embryonic Stem Cells: A Practical Approach. IRL Press, Oxford, E. J. Robertson, ed. pp 153-182, 1987). Therefore, on the assumption that the transgene has no direct effect upon viability, 20% of embryos reconstituted from unselected, injected TNT cells and cultured to blastocyst will be stably transgenic. The numbers of final recipient animals required may be further reduced by employing biopsy procedures prior to final transfer (step 4a above), although there will be a concomitant reduction in viability.

Culture to blastocyst and final embryo transfer

The average proportion of injected and transferred pronuclear ungulate eggs which generate transgenics is below 1.0% (Clark *et al*, "Germline Manipulation: Applications in Agriculture and Biotechnology" In: *Transgenic Animals*. Academic Press, London F. Grosveld and G. Kollias, eds. pp 247-270 (1992)). This means that

for a single cattle transgenic project, several hundred embryo transfers are required, involving similar numbers of recipient females. Hence several hundred cattle need to be maintained for a period of 15 months (embryo transfer to weaning). With TNT cells, preselection of transgenic clones would mean that 100% of liveborn animals would be transgenic, and the only losses would be due to the proportion of TNT-derived blastocysts transferred to final recipients which fail to develop to term. Using *in vivo* culture in temporary recipients, preliminary data suggest that development to blastocyst strongly correlates with development to term (Table 1), raising the possibility that the numbers of recipients required can be reduced by an order of magnitude using the TNT system. These advantages can be gained with quite early passage TNT cells (passages 4-6 depending upon transfection method).

Table 1. Fusion, development to morulae/blastocyst and development to term of reconstituted embryos from TNT and 16 cell blastomere nuclei

Cell Line	Passage	Fused	Number transferred	Lambs
TNT/2	P1	15	2	0
TNT/3	P1	16	2	0
TNT/4	P2	23	1	stillborn
TNT/4	P3	49	2	2
TNT subtotal		103	7	2
16 Cell		72	9	4

Gene targeting

Sheep TNT cells at passage 3 and beyond grow rapidly in culture, with a doubling time of approximately 24 hours. Gene targeting regimes similar to those used for ES cells require 10^7 cells for the initial electroporation, although in principle this can be reduced. These numbers

of TNT cells are available by passage 4 (approximately 23 cell divisions). Development to term from TNT cells at passage 3 has already been achieved. Subsequent selection will involve a further 14-20 cell divisions (equivalent to passage 7-9). It is anticipated that if TNT nuclei at later passages (for example, passage 10) are still able to support development to term, then it will become possible to do gene targeting in ungulates. Procedures using cell surface markers followed by cell sorting could even reduce the passage number to 5 or 6.

Preferred features for each aspect of the invention are as for each other aspect, *mutatis mutandis*.

The invention will now be illustrated by the following examples. The examples refer to the drawings, in which:

FIGURE 1 shows the appearance of TNT/4 cells at passage 4 (circled) growing on an inactivated fibroblast feeder layer (arrowed). TNT cells have an enlarged, flattened, epithelial phenotype, contrasting sharply with ES cells, which are small, rounded and have a high nucleus:cytoplasm ratio; and

FIGURE 2 shows a lamb (left) derived from fusion of a Welsh Mountain-derived TNT cell to a Scottish Blackface oocyte, followed by transfer to a Scottish Blackface foster mother (right). Lamb and foster mother show characteristic markings of Welsh Mountain (TNT/4 genotype) and Scottish Blackface (recipient oocyte genotype) respectively.

EXAMPLE 1: Isolation of Sheep TNT Cell Lines

The oestrus cycles of Welsh Mountain ewes were

synchronised with progesterone impregnated sponges. Ewes were then superovulated with 2 daily injections of equine follicle-stimulating hormone. Ovulation was induced with a single injection of gonadotropin release hormone (gnRH) and animals were artificially inseminated by laparoscopy using semen from East Friesland (white) rams. Embryos were subsequently flushed from the reproductive tract using standard surgical techniques. Embryo culture technique is very similar to that used in attempts at ES isolation, with 2 significant differences: (1) slightly later stage embryos, (blastodermic vesicles) are entered into culture and (2) undifferentiated ES-like early colonies are allowed to differentiate to generate rapidly dividing, permanent epithelial cell lines.

In contrast to Strelchenko *et al.*, *loc. cit.*, and Sims & First, *loc. cit.*, who used earlier stages, forty embryonic discs (EDs) at days 9 and 10 were dissected free of trophectoderm and cultured in groups of three to ten on inactivated primary mouse fibroblasts in 24 well culture plates. Culture medium was Glasgow's modified eagles medium (GMEM; Gibco, UK) supplemented with 10% foetal bovine serum (Gibco, UK) and 1000U/ml of the anti-differentiation agent, leukaemia inhibition factor (LIF). EDs were disaggregated by mild trypsinisation after 3-7 days (ie after attachment and evidence of outgrowth) and passaged onto a fresh feeder layer (passage 1). Within 7-10 days a proportion of wells showed small foci of undifferentiated cells. These were picked and passaged onto fresh feeders again in 24 well plates (passage 2 or P2). P2 cells expanded rapidly, losing their small, round, undifferentiated phenotype (Figure 1). At confluence, P2s were passaged into a single 25cm^2 flask. four such lines were isolated and designated TNT/1 through 4. Three lines were frozen at passage 4.

The cell line TNT/4 was thawed and then cultured for a further 6 passages. Cells maintained a stable phenotype and failed to generate embryoid bodies when cultured in suspension. This differs markedly from ES cells and from the bovine cells of Strelchenko *et al., loc. cit.*, which are characterised by their ability to form embryoid bodies and their undifferentiated morphology. There was no apparent change in phenotype when cultured in the absence of feeders.

EXAMPLE 2: Generation of Lambs from TNT Nuclei

Recipient oocytes were obtained following superovulation as per donors above, with the exception that no artificial insemination occurred. Unfertilised (Black Welsh Mountain and Scottish Blackface) MII oocytes were recovered in phosphate buffered saline (PBS) by flushing from the oviduct 31-33 h after gnRH injection. Recovered oocytes were washed in OCM medium (Gibco, UK) and transferred to medium TCM 199 (Gibco, UK). To remove the chromosomes (enucleation), oocytes were placed in TCM 199 containing 10% FBS, 7.5μg/ml cytochalasin B (Sigma, UK) and 5.0μg/ml Hoechst 33342 (Sigma, UK) at 37°C for 20 minutes. A small amount of cytoplasm from directly beneath the first polar body was removed with a finely drawn pipette and enucleation was confirmed by exposing the aspirated cytoplast to UV light and checking for the presence of a metaphase plate.

Oocytes were activated in a chamber consisting of two parallel platinum electrodes arranged 200 μm apart in a glass petri dish 9 cm in diameter. Oocytes were placed between the electrodes in 80 μl of activation medium (0.3 M mannitol, 0.1 mM $MgSO_4$, 0.001 mM $CaCl_2$) in distilled water. Activation was induced by a single DC pulse of 1.25 kV/cm for 80 μs.

Embryos were reconstructed by cell fusion (nuclear transfer from TNT cells) between 6 and 12 hours post activation (Campbell *et al.*, *Biol. Reprod.* **50** 1385-1393 (1994)). Fusion was carried out in the same chamber described above for activation, by application of a single AC pulse of 3V for 5 s followed by 3 DC pulses of 1.25 kV/cm for 80μs in activation medium. Reconstructed embryos were cultured in TCM 199 plus 10% FBS and 7.5 μg cytochalasin B for 1 hour at 37°C in 5% CO_2, were double embedded in 1% and then 1.2% agar (Difco) and then transferred to the ligated oviduct of unsynchronised Black Welsh Mountain ewes (temporary recipients for *in vivo* culture). After 6 days, recipient ewes were killed and the embryos were retrieved by flushing with PBS. Embryos were dissected from the agar. Those which had developed to morula or blastocyst stages were transferred to final synchronised recipients.

Seven reconstituted embryos were transferred into five final recipients, three of which returned to oestrus. The remaining two ewes yielded two liveborn lambs which displayed the white markings characteristic of the TNT lines (Table 1). One of these animals died shortly after birth and the remaining animal was healthy at three months of age (Figure 2) and survived until it was six months old, when it died from a kidney problem.

EXAMPLE 3: Transfection of foreign DNA into TNT cells

Foreign DNA has been introduced to TNT cells by: (i) electroporation (ii) by lipofection and (iii) by Ca-phosphate precipitation. Transfection was monitored either by histological staining for marker gene expression or by quantitation of fluorescence catalysed by a luciferase reporter gene in transient assays. The

test plasmid used in transient transfections comprised a PGK promoter driving the β-galactosidase marker. This gene's activity can be detected by staining with the chromogenic substrate 5-bromo-4-chloro-3-indoyl-β-D-galactosidase (X gal; Sigma, Poole, UK) (Bondi *et al.*, *Histochem* 76: 155-8 (1982)). For stable transfection a variety of text plasmids were used which linked a selectable marker with either the β-gal or luciferase reporter genes. The selectable marker utilised an SV40 promoter to drive expression of the neomycin phosphotransferase gene (*neo*) which confers resistance to the neomycin analogue G418.

All methods were functional for the introduction of DNA to TNT cells, although calcium phosphate precipitation was associated with a high incidence of syncytia (cell fusion) and was, for this reason, deemed unsuitable for further investigation. Lipofection produced the greatest number of cells staining both per μg DNA and per cell number exposed and we are currently concentrating on the optimisation of lipofection protocols for this cell type. Stable transfection has been achieved following electroporation but has not yet been tested following lipofection.

(i) Electroporation

Electroporations were carried out in 800 μl volumes containing 1 to 2 x 10^6 cells and 1.0 μg of circular (for transient transfection) or linear (for stable transfection) test plasmid DNA. Electroporations were carried out using a GENE PULSER™ machine (Bio Rad, Herts, UK) and in all other respects was as described elsewhere (Thompson *et al.*, *Cell* 56: 313-21 (1989)). The best conditions tested yielded only 59 cells per 2 x 10^5 cells.

Table 2. Numbers of stained cells per 25 cm^2 flask

μFD	Volts	Colonies staining
125	120	27
125	250	113
125	450	0
250	120	31
250	250	34
250	450	0
500	120	41
500	250	115
500	450	0

ii) Lipofection

Lipofection techniques involve the packaging of DNA into lipid particles whose properties promote fusion with cells membranes. All lipofections were performed in OPTIMEM™ medium (Life Technologies, Paisley, UK) and LIPOFECTAMINE™ (Life Technologies, Paisley, UK). 2 x 10^5 cells were plated onto one 6cm dish for each treatment. Following attachment, medium was replaced with OPTIMEM containing 1.5μg DNA and 10 μg LIPOFECTAMINE, and was incubated for 1-18 hours at which time OPTIMEM was replaced with normal TNT medium (treatment a). In an alternative treatment, 2 volumes of TNT medium were added to each plat 1-18 hours after addition of OPTIMEM/DNA/LIPOFECTAMINE, in this instance, leaving the OPTIMEM/DNA/LIPOFECTAMINE *in situ* (treatment b). Twenty-four to 48 hours later, cells were screened for β-gal or luciferase expression. The best conditions tested yielded >1000 cells staining per 2 x 10^5 cells.

Tabl 3. Numbers of stained cells per field following lipofection

Time in LIPO/ DNA/OPTIMEM	LIPO/DNA/OPTIMEM removed (a)	diluted (b)
1 hour	7	30
2 hours	35	65
4 hours	140	150
18 hours	250	250

CLAIMS

1. An animal cell line derived from an embryonic disc of an ungulate blastodermic vesicle, or the equivalent tissue of an embryo at an equivalent stage in non-ungulate species, cells of which cell line are totipotent for nuclear transfer.

2. An isolated animal cell which is derived from an embryonic disc of a ungulate blastodermic vesicle, or the equivalent tissue of an embryo at an equivalent stage in non-ungulate species, and which is totipotent for nuclear transfer.

3. A cell line or cell as claimed in claim 1 or 2, wherein the animal is a placental mammal.

4. A cell line or cell as claimed in claim 1 or 2, wherein the animal is an ungulate.

5. A cell line or cell as claimed in claim 1 or 2, wherein the animal is a cow or bull, sheep, goat, water buffalo, camel or pig.

6. A cell line or cell as claimed in claim 1 or 2, which contains one or more transgenes.

7. A process for the preparation of an animal, the process comprising reconstituting an animal embryo by nuclear transfer from a TNT cell from a cell line as claimed in claim 1, or from a TNT cell as claimed in claim 2, allowing the embryo to develop to term and optionally breeding from the animal so formed.

8. A process as claimed in claim 7, wherein the TNT cell is genetically modified prior to embryo reconstitution.

9. A process as claimed in claim 8, wherein the TNT cell is genetically modified by electroporation, by viral transfection or by lipofection.

10. A process for the isolation of cells which are totipotent for nuclear transfer (TNT cells), the process comprising explanting at least part of the embryonic disc of an animal embryo at the blastodermic vesicle stage in the case of an ungulate, or the equivalent tissue of an embryo at an equivalent stage in non-ungulate species, allowing the development of an ES-like colony of undifferentiated cells until they have acquired an enlarged, more epithelial phenotype and entering a cell or cells from such a colony into reproductive culture until required.

FIG. 1.

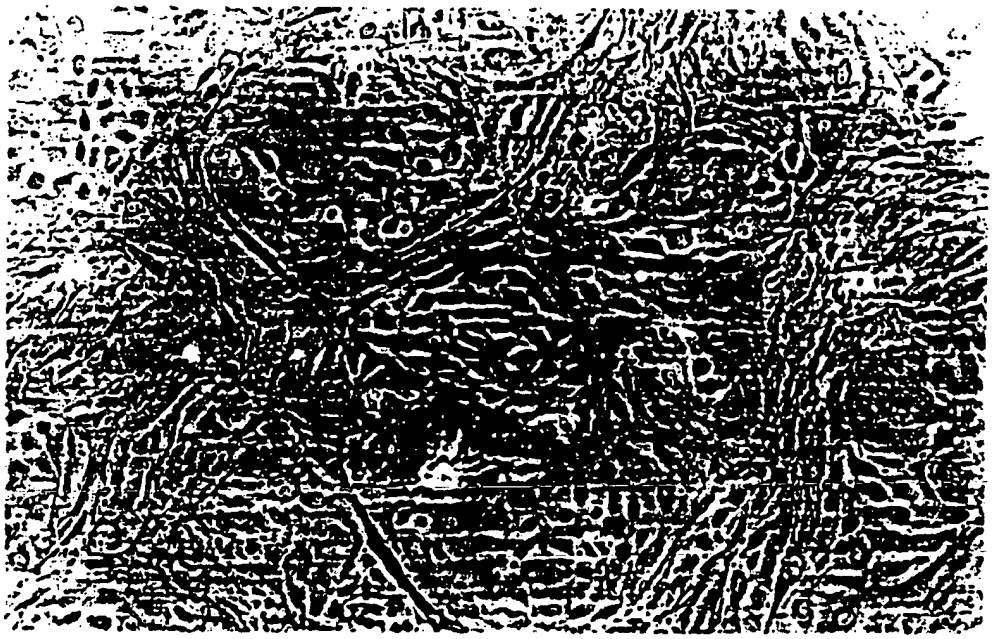
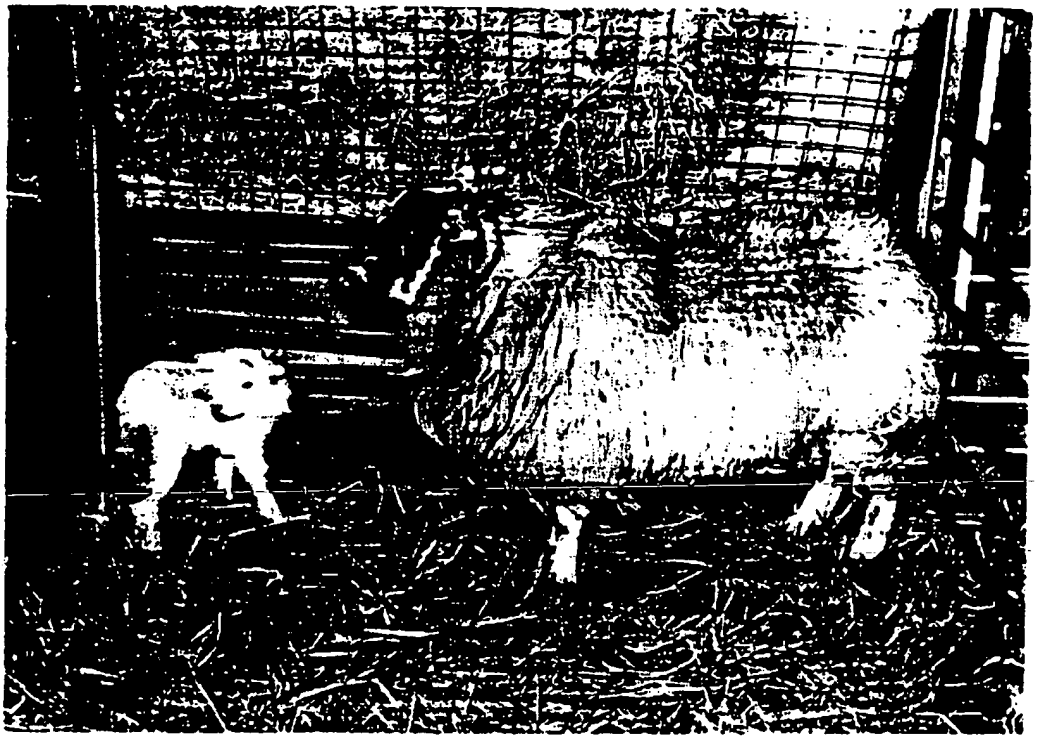


FIG. 2.



INTERNATIONAL SEARCH REPORT

Int .onal Application No
PCT/GB 95/02095

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/06 C12N5/10 A01K67/027

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ANNUAL CONFERENCE OF THE INTERNATIONAL EMBRYO TRANSFER SOCIETY, MELBOURNE, VICTORIA, AUSTRALIA, JANUARY 9-11, 1994. THERIOGENOLOGY 41 (1). 1994. 301., STICE S ET AL 'Bovine pluripotent embryonic cells contribute to nuclear transfer and chimeric fetuses.' cited in the application see the whole document --- -/--	1-10

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 December 1995	0 8. 01. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Moreau, J

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ANNUAL CONFERENCE OF THE INTERNATIONAL EMBRYO TRANSFER SOCIETY, MELBOURNE, VICTORIA, AUSTRALIA, JANUARY 9-11, 1994. THERIOGENOLOGY 41 (1). 1994. 304., STRELCHENKO N ET AL 'Bovine embryonic pluripotent cell lines derived from morula stage embryos.' cited in the application see the whole document ---	1-10
X	REPRODUCTION FERTILITY AND DEVELOPMENT 6 (5). 1994. 553-562. , FIRST N L ET AL 'Systems for Production of Calves from Cultured Bovine Embryonic Cells.' see the whole document ---	1-10
X	PROC. NATL. ACAD. SCI. U. S. A. (1994), 91(13), 6143-7, 1994 SIMS, MICHELLE ET AL 'Production of calves by transfer of nuclei from cultured inner cell mass cells' cited in the application see the whole document ---	1-10
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X	BIOLOGY OF REPRODUCTION, vol. 50, no. 4, April 1994 pages 935-939, KEEFER C.I. 'Bovine Inner Cell Mass Cells as Donor Nuclei in the Production of Nuclear Transfer Embryos and Calves' cited in the application see the whole document ---	1-10
P,X	WO,A,94 26884 (BIOTECHNOLOGY RESEARCH AND DEVELOPMENT CORPORATION) 24 November 1994 see the whole document ---	1-10
P,X	WO,A,95 16770 (ABS GLOBAL INC.) 22 June 1995 see the whole document --- -/--	1-10

INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/GB 95/02095

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	EXPERIMENTAL BIOLOGY 95, PART II, ATLANTA, GEORGIA, USA, APRIL 9-13, 1995. FASEB JOURNAL 9 (4). 1995. A833., STRELCHENKO N ET AL 'Differentiation of bovine pluripotential stem cells in vitro.' see the whole document ---	1-10
P,X	ANNUAL CONFERENCE OF THE INTERNATIONAL EMBRYO TRANSFER SOCIETY, CALGARY, ALBERTA, CANADA, JANUARY 8-10, 1995. THERIOGENOLOGY 43 (1). 1995. 181., CAMPBELL K ET AL 'Production of live lambs following nuclear transfer of cultured embryonic disc cells.' see the whole document -----	1-10

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INTERNATIONAL SEARCH REPORT

Information on patent family members

In' ional Application No
PCT/GB 95/02095

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9426884	24-11-94	AU-B-	7019394	12-12-94
WO-A-9516770	22-06-95	AU-B-	1439295	03-07-95